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Differences in Proinflammatory Cytokines and Monocyte Subtypes in Older as Compared With Younger Kidney Transplant Recipients

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Background. The number of elderly patients with end-stage kidney disease requiring kidney transplantation continues to grow. Evaluation of healthy older adults has revealed proinflammatory changes in the immune system, which are posited to contribute to age-associated illnesses via "inflamm-aging." Immunologic dysfunction is also associated with impaired control of infections. Whether these immunologic changes are found in older kidney transplant recipients is not currently known, but may have important implications for risk for adverse clinical outcomes. **Methods.** Three months after transplant, innate immune phenotype was evaluated by flow cytometry from 60 kidney transplant recipients (22 older [≥60 years] and 38 younger [<60 years old]). Multiplex cytokine testing was used to evaluate plasma cytokine levels. Younger patients were matched to older patients based on transplant type and induction immune suppression. **Results.** Older kidney transplant recipients demonstrated decreased frequency of intermediate monocytes (CD14++CD16+) compared with younger patients (1.2% vs 3.3%, $P = 0.007$), and a trend toward increased frequency of proinflammatory classical monocytes (CD14++CD16–) (94.5% vs 92.1%) ($P = 0.065$). Increased levels of interferon-gamma (IFN-γ) were seen in older patients. **Conclusions.** In this pilot study of kidney transplant recipients, we identified differences in the innate immune system in older as compared with younger patients, including increased levels of IFN-γ. This suggests that age-associated nonspecific inflammation persists despite immune suppression. The ability to apply noninvasive testing to transplant recipients will provide tools for patient risk stratification and individualization of immune suppression regimens to improve outcomes after transplantation.

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The proportion of elderly individuals with end-stage renal disease (ESRD) has increased dramatically, from 36% of incident ESRD cases in 1985 to nearly half in 2013, constituting the majority of the growth in the ESRD patient population.[1] Elderly transplanted patients experience a higher rate of infection and risk of death due to infection, as well as a decreased rate of acute rejection.[1-4] Furthermore, when these patients develop rejection, they are less likely to respond favorably to treatment.[1,5]

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E.L. and M.R. participated in writing of the paper, performance of the research, research design, and data analysis. T.S., V.G., G.S., and Y.K. participated in research design and performance of the research. S.V. participated in data analysis. B.A., E.L., E.H., S.B., T.P., and G.B. participated in writing of the article and performance of the research. E.R. and J.S. participated in research design, writing of the article, and data analysis.

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These findings suggest a critical role for immune dysfunction in elderly kidney transplantation outcomes. Immunosenescence, the decline in immune function with age, is associated with greater susceptibility to infectious diseases and reduced vaccination efficacy, and is characterized by changes in both innate and adaptive immune cell subsets and function.[6,7] Age-related deficiencies in the innate immune system include decreased NK cell cytokine production and cytotoxicity and impaired phagocytosis and reactive oxygen species production by monocytes/macrophages.[5,7–9] Other aspects of immune dysfunction include decrease in Toll-like receptors (TLR) including TLR4 (also known as CD284) expression on dendritic and other types of innate immune cells, especially important in monocytes given their high levels of expression of this receptor fitting their role as the major responders to bacterial lipopolysaccharide (LPS).[10,11]

The immunologic dysfunction of aging is also associated with CMV seropositivity and higher levels of proinflammatory cytokines including interferon (IFN)-gamma.[7,12] The age-related increase in proinflammatory cytokines is part of a chronic low-grade inflammatory state known as "inflamm-aging," which is itself associated with chronic age-related diseases, such as type II diabetes and cardiovascular disease.[9,13,14] An increase in the classical proinflammatory monocyte subtype has also been associated with inflammation and chronic disease, and may be exacerbated in the setting of a proinflammatory environment.[15] Monocytes are an important cell type to study in the analysis of inflammation because they are attracted to sites of inflammation and after activation secrete cytokines that can perpetuate the cycle of inflammation.[16,17] CD14 and CD16 expression is used to differentiated human monocyte subsets into classical (CD14++/CD16−), intermediate (CD14++/CD16+), and nonclassical (CD14+/CD16++).[18] Although the roles of the different monocyte subsets in humans is not clearly defined, CD16++ nonclassical monocytes display a mixture of proinflammatory and anti-inflammatory tissue-repair properties, whereas the classical and intermediate CD14 ++ monocyte subsets play a major role in phagocytosis and inflammation.[19,20]

It is not known what impact the administration of immune suppression medications may have on the proinflammatory effect of increased patient age, or how this may impact progression of age-related diseases after transplantation.

Receipt of deceased donor compared with living donor transplants may also impact morbidity and mortality in older patients.[1,4] The combination of immune dysfunction and inflamm-aging may explain the lack of resilience seen in older transplant patients experiencing infection and rejection.

Reaching a better understanding of immune system function in the setting of immune suppression in the older compared with the younger transplant recipient may not only lead to insights to the mechanism of poorer posttransplant outcomes in older patients, but also to the ability to individualize immune suppression regimens and avoid over immune-suppression in the older and more immunosenescent transplant recipient. These analyses should also take into account potential differences in donor type on the older patient. To examine age-related changes in the innate immune system in the context of immune suppression, we present here an investigation of innate immune cell phenotypes and cytokine production in older and younger kidney transplant patients.

MATERIALS AND METHODS

Patients and Samples

Patients undergoing kidney transplantation at Ronald Reagan Medical Center were enrolled in this observational study, which was approved by the UCLA Institutional Review Board. All patients signed informed consent. Blood was collected for peripheral blood mononuclear cell (PBMC) isolation at 3 months after transplantation. We identified 22 older patients, older than 60 years, who had PBMC available for analysis. These older patients were matched with 38 patients between the ages of 30 and 51 years, matched on deceased versus living donor and lymphocyte-depleting versus non–lymphocyte-depleting induction with basiliximab induction therapy, for a total cohort of 60 patients.

PBMC and plasma were isolated using previously published techniques,[21,22] and frozen for storage until batched analysis could be performed.

Patients at increased risk for rejection, defined as panel-reactive antibodies greater than 20%, history of donor specific antibodies, positive crossmatch, cold ischemia time longer than 24 hours, or donation after cardiac death, received induction with antithymocyte globulin (ATG). Patients not meeting these criteria received basiliximab for induction. Maintenance immunosuppression was performed with tacrolimus, mycophenolate mofetil, and prednisone. Mycophenolate mofetil and prednisone doses were similar in each patient, and tacrolimus was started at equivalent doses per kg body weight with equivalent target drug levels in each patient following the UCLA protocol. Patients received valganciclovir prophylaxis for cytomegalovirus for 6 months for high risk (donor positive, recipient negative) and 3 months for low risk (recipient positive) patients. Cotrimoxazole sulfate was administered for the first year after transplantation.

Flow Cytometry

Viable cells were identified using a fluorescent live/dead marker (Life Technologies). Innate cell subsets were evaluated using a cocktail of fluorochrome-conjugated antibodies. CD14 and CD16 were used to define monocyte subsets as shown in Figure S1, http://links.lww.com/TXD/A64.[18,23] CD284 (Toll like receptor 4) to define ability to recognize LPS.[24] Antibodies were obtained from either BD Biosciences or Biolegend. Fluorescence from viable cells was measured by the BD LSRFortessa (BD Biosciences) with analysis via FCS Express software (DeNovo Software).

Multiplex Cytokine Analysis

The assay was performed in the UCLA Immune Assessment Core. Human 38-plex magnetic cytokine/chemokine kits were purchased from EMD Millipore and used per manufacturer's instructions. This technique has been previously validated in our laboratory to demonstrate excellent reproducibility in multiplex cytokine analysis.[25] The following analytes were detected: G-CSF, GM-CSF, IFN-gamma, IFN-a2, IL-1beta, IL-1Ra, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 (p40), IL-15, IL-17A, MCP-1, MIP-1alpha, MIP-1beta, CD40L, MDC, TNF-alpha, EGF, FGF-2, GRO, Eotaxin, Fractalkine, FLT-3L, and VEGF. Fluorescence was quantified using a Luminex 200 instrument.

TABLE 1.

Demographic characteristics of older and younger kidney transplant recipients matched on transplant type and induction

	Younger, n = 37	Older, n = 23
Age (median), y	43 (34-51)	67 (60-80)
Male sex	22 (59.5)	17 (73.9)
White race	25 (67.6)	15 (65.2)
Hispanic	15 (40.5)	8 (34.8)
Dialysis pretransplant	27 (72.2)	21 (91.3)
CMV antibody positive	26 (70.3)	18 (78.3)
CMV mismatch (D+/R–)	5 (13.5)	4 (17.4)
Induction, ATG	11 (29.7)	7 (30.4)
Deceased donor	17 (45.9)	10 (43.5)
Time post transplant (median), d	87	89

Numbers followed by a percentage as applicable.

Clinical Data Collection

Data were collected via review of the clinical record on immunosuppression induction type, living versus deceased donor, dialysis receipt and time on dialysis, pretransplant diagnosis of diabetes mellitus, CMV antibody status, history of rejection using Banff criteria, and history of infection or CMV viremia using standard definitions, and death in the first year after transplantation.[26-28] Urinary tract infections were not considered given the difficulty in distinguishing colonization from invasive disease. Severe infection was defined as requiring intravenous antibiotic treatment and/or leading to extension of hospital stay or death.

Statistical Analysis

Statistical analysis was performed using JMP Pro 11 (SAS Software). Differences between continuous values were compared by nonparametric 2-sample test (Mann-Whitney *U* test), whereas differences between categorical variables were compared by Fisher exact test. Multivariate logistical rejection was performed by stepwise regression using minimum BIC as a stopping rule. After log transformation of cytokine values to mitigate influence of outliers, heat map was created by complete linkage hierarchical clustering and principal component analysis created using R project version 3.3.3 software.

RESULTS

Patient Characteristics in Older and Younger Cohorts

Twenty-three patients older than 60 years who had undergone kidney transplantation at our center were matched based on transplant type (living vs deceased) and induction (ATG vs basiliximab) with 37 patients aged 30 to 51 years (Table 1). These older and younger patients had similar proportions of sex, race, and ethnicity. Pretransplant diabetes mellitus and receipt of dialysis tended to be more common in the older patients, but these trends also did not reach statistical significance ($P = 0.107$ and $P = 0.103$, respectively) (Table 1). Acute rejection in the first year did not differ significantly by patient group, with an incidence of 16.2% in the younger patients and 8.7% in older patients ($P = 0.698$). There was a trend toward increased incidence of CMV viremia in the older patient group, with 24.3% incidence in the younger and 47.8% in the older patients ($P = 0.091$). Incidence of non–urinary tract infection invasive infection in the first year posttransplant were similar in this cohort, with 6 infections in the younger group (16.2%) and 4 in the older group (17.4%).

Proinflammatory Innate Immune Cell Phenotypes and Patient Age

The innate immune cell phenotypes of the older and younger kidney transplant recipients were analyzed at 3 months posttransplant, the earliest time point at which sufficient numbers of cells in the ATG-induced patients could be obtained. Compared with the younger kidney transplant recipients, the older kidney transplant recipients had a decreased frequency of intermediate (CD14++/CD16+) monocytes (3.27% vs 1.16%, $P = 0.007$). There was a trend toward increased levels of classical (CD14++/CD16–) monocytes in the older compared with younger patients which did not reach statistical significance (94.5% vs 92.1%, $P = 0.065$) (Figure 1). The nonclassical monocyte subset did not differ significantly by patient age (2.94% in older vs 3.20% in younger patients, $P = 0.398$).

Frequency of dendritic cells ($CD14^{dim}CD16–$) was increased in older patients (5.5% vs 2.8%, $P = 0.021$). Frequency of CD56+ cells was trended toward increase in older versus younger patients, but did not reach statistical significance (15.0% in older vs 11.0% in younger, $P = 0.118$).

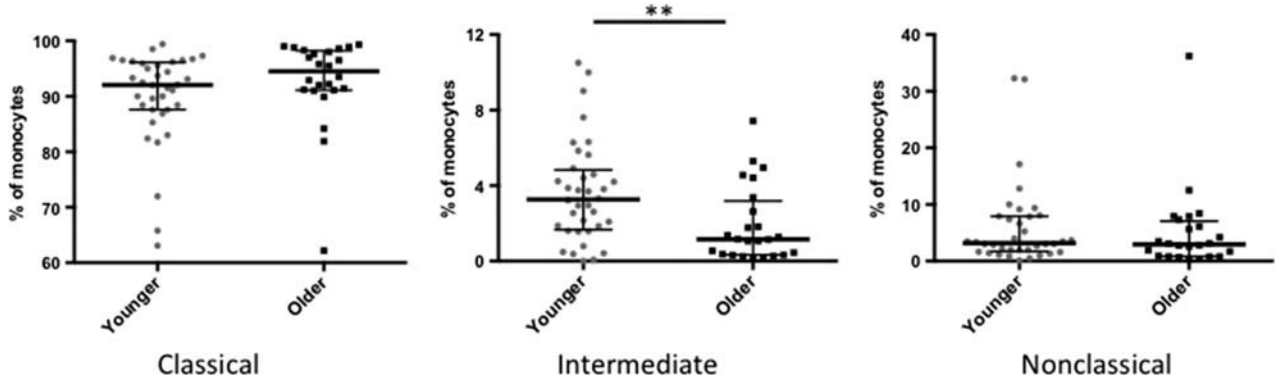

FIGURE 1. Frequency of monocyte subtypes by patient age (younger vs older). PBMC were analyzed for classical (CD14++/CD16–), intermediate (CD14++/CD16+), and nonclassical (CD14+/CD16++) monocytes, expressed as a percentage of the total number of monocytes. Each dot corresponds to a sample; bars indicate median. $^{**}P < 0.01$ by nonparametric test.

Immune Dysfunction-Related Innate Immune Cell Phenotypes and Patient Age

Older patients demonstrated decreased frequencies of CD284 (TLR4)-expressing monocytes (60.7% vs 88.9%, $P = 0.040$) (Figure 2A). A statistically significant difference was also seen in the classical CD284+ monocyte subset (72.0% for older vs 96.5% for younger patients, $P = 0.039$). For intermediate and nonclassical subtypes, a similar trend was observed that did not reach statistical significance (intermediate, 86.9% for older vs 97.1% for younger, $P = 0.075$, and nonclassical, 65.9% for older vs 88.4% for younger, $P = 0.189$) (Figure 2B). Innate immune cell phenotypes that were not associated with patient age included $CD14^{high}$KLRG1+, $CD14^{dim}$KLRG1+, and CD56+ KLRG1+.

Innate Immune Cell Phenotypes and Non–Age-Related Clinical Characteristics

We also evaluated whether innate immune cell phenotypes were associated with clinical characteristics other than age. Recipients of deceased donor transplants demonstrated decreased numbers of CD14+ CD284+ cells (59.4% for deceased vs 89.1% for living donor, $P = 0.031$) (Figure 2C). A statistically significant difference was also seen in all CD284+ monocyte subsets: classical (66.0% for deceased vs 96.2% for living donor, $P = 0.023$), intermediate (86.3% for deceased vs 97.9% for living donor, $P = 0.015$), and nonclassical (57.9% for deceased vs 89.0% for living donor, $P = 0.003$) (Figure 2D).

CMV seropositivity and CMV viremia did not have an impact on these innate immune cell subtypes (data not shown).

No associations between innate immune cell phenotypes and invasive infection posttransplant were seen. No significant differences in innate immune cell phenotypes were seen by other clinical characteristics, including history of dialysis, pretransplant diabetes mellitus, and incidence of acute rejection.

Cytokine Production and Patient Age

To evaluate the overall proinflammatory profile of transplant recipients, we measured a panel of plasma cytokines and chemokines and visualized them by means of hierarchical clustering to identify expression trends. Proinflammatory cytokines tended to cluster with increased patient age (Figure 3A). To evaluate the overall ability of these markers to distinguish between elderly and nonelderly patients, principal component analysis was performed to evaluate differences in cytokine expression patterns between patient groups but did not reach statistical significance ($P = 0.065$ by 2-sample t test). When analyzed individually, plasma levels of IFN-gamma significantly increased with patient age ($P = 0.020$) (Figure 3B), but there were no other significant associations with other cytokine levels and patient age (data not shown). There were no significant associations between cytokine levels and pretransplant characteristics, invasive infection posttransplant, or CMV viremia (data not shown).

DISCUSSION

Recent research has shown that the both the innate and adaptive immune systems become dysregulated with age, with immunosenescence and inflammaging contributing to increased morbidity and mortality due to infection, poorer responses to vaccination, and the progression of diseases associated with chronic inflammation.[7] To determine whether these deleterious changes persist in immunosuppressed transplant patients, we evaluated innate immune cell phenotypes in older and younger kidney transplant recipients. We found

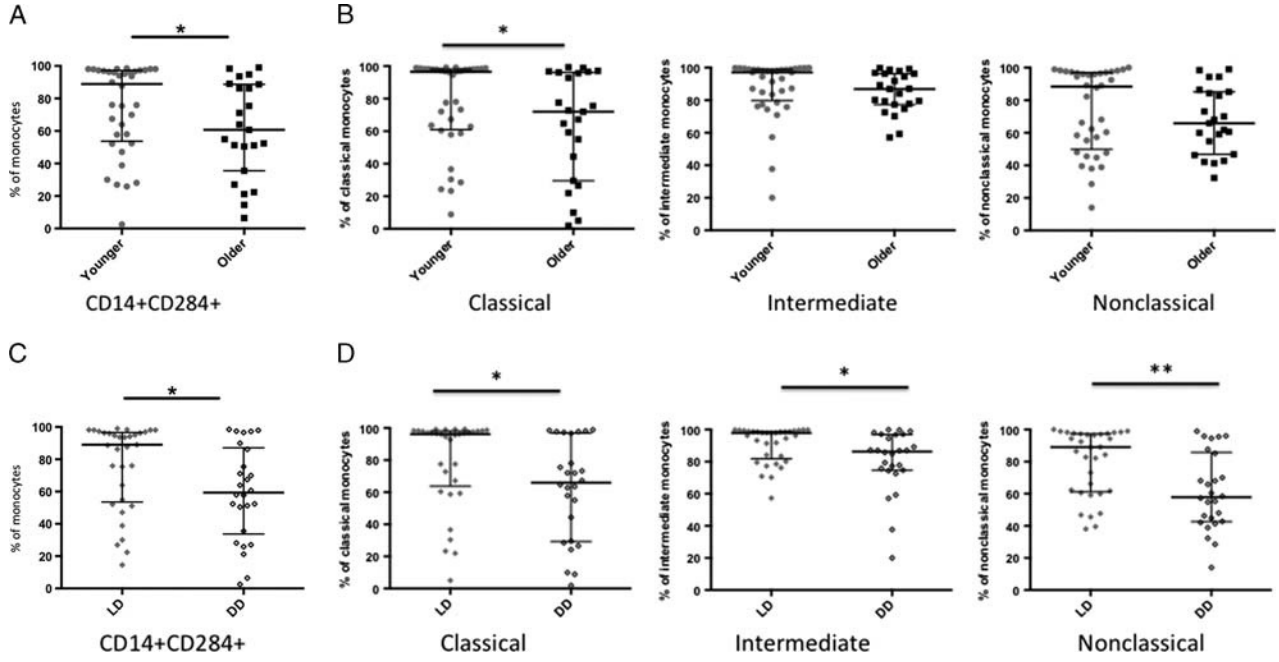

FIGURE 2. Frequency of monocyte subtypes expressing TLR4. A, Frequency of CD14+ monocytes expressing TLR4 (CD284) by patient age (younger vs older). B, Frequency of monocyte subtypes (classical, intermediate, and nonclassical) expressing TLR4 (CD284) by patient age (younger vs older). C, Frequency of CD14+ monocytes (right panel) expressing TLR4 (CD284) by donor type (living (LD) vs deceased donor (DD)). D, Frequency of monocyte subtypes expressing TLR4 (CD284) by donor type. Each dot corresponds to a sample; bars indicate median. *$P < 0.05$ by nonparametric test.

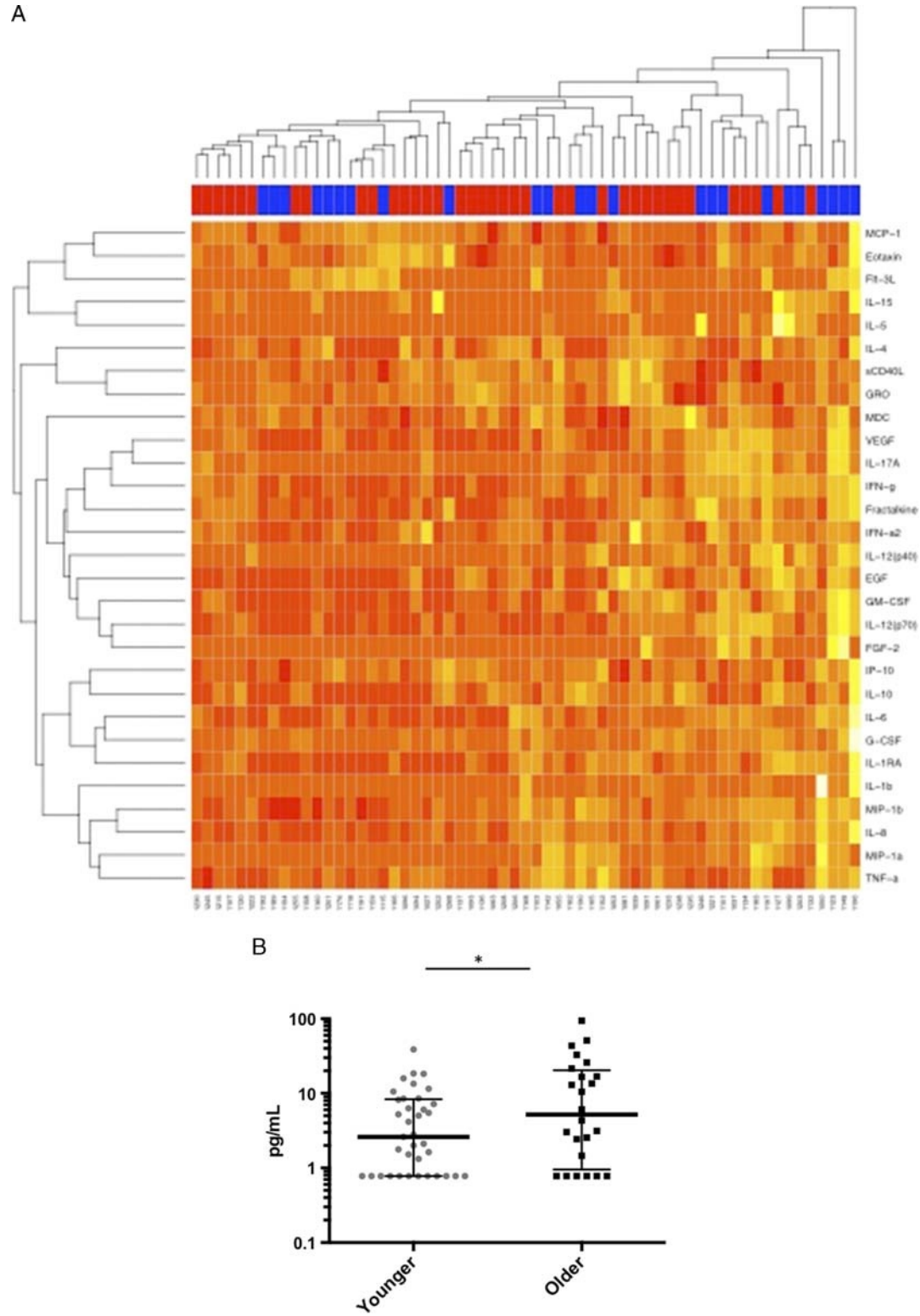

FIGURE 3. Analysis of cytokine expression by patient age. A, Heatmap by complete linkage hierarchical clustering of log-transformed concentration of cytokines in the plasma of kidney transplant recipients. Yellow indicates higher expression levels, and red indicates lower expression levels. Older patients (≥ age 60 years) indicated by blue blocks, and younger patients by red blocks, on the right hand side of the figure. B, Box and whiskers plot of IFN-gamma expression by older (black squares) and younger (grey circles) patients. Bar shows median and whiskers show interquartile range. *$P < 0.05$ by nonparametric test and **$P < 0.01$.

that the older kidney transplant recipients had a decreased frequency of intermediate CD14++/CD16+ monocytes compared with younger patients matched for induction immune suppression and deceased versus living donor type, and a trend toward an increase in classical monocytes. Intermediate monocytes are enriched for markers of monocyte activation,

including HLA-DR, CD11b, and CD115, display high phagocytic activity, have higher T-cell stimulatory ability compared with classical monocytes, and are thought to include dendritic cells precursors.[19] In addition, intermediate monocytes are associated with chronic kidney disease and endothelial wall binding.[29] Overall, this difference in subtype frequency suggests a possible mechanism behind poor response to infection in older patients. In contrast, there was a trend toward higher levels of proinflammatory cytokines in the older patients, which is in line with the increase in the more proinflammatory classical monocyte subset. We also observed a decreased frequency of CD14+ cells expressing TLR4, suggesting a reduced capability to control bacterial infections. These results indicate that a degree of age-associated immune dysfunction can be detected in older compared with younger patients on identical regimens of induction and posttransplant immunosuppressive therapy.

Other innate immune cell phenotypes were not associated with age, and there were no associations between innate immune phenotypes and invasive infection posttransplant.

Previous studies have found skewing toward the proinflammatory M1 macrophage subset in older healthy individuals.[17] This is in line with our observation toward an increase in classical monocytes in older patients, because this subset is thought to be prone to M1 over M2 differentiation.[30] These observations may be explained by age-related changes in proinflammatory cytokines, particularly IFN-gamma, which both activates and is released by monocytes.[17] We also found increased levels of IFN-gamma in the older patients.

LPS is another major regulator of classical monocyte activation; however, we found that older patients tended to have decreased frequencies of CD14+ CD284+ innate immune cells overall, indicating an impaired response to bacterial infection in older patients and a smaller role for LPS in promoting monocyte activation compared to proinflammatory cytokines. The decreased frequencies of innate immune cells capable of recognizing LPS may also explain why older patients are at increased risk of infection and death due to infection after transplantation.[2,31] Although the trend did not reach statistical significance, likely due to the overall low rate of infection in our cohort, the older kidney transplant recipients in our study tended to have increased risk of invasive infection or CMV viremia posttransplant. Monitoring of this subset may provide an approach for risk stratification of risk for bacterial infection in older transplant recipients. Interestingly, patients receiving kidneys from deceased donors also demonstrated decreased frequency of CD14+ CD284+ innate immune cells, which may at least partially explain the mechanism behind poorer clinical outcomes in this patient group.

The main limitation of this study is the small size of the study cohort, which may accentuate any heterogeneity found between patients sampled. However, both age groups received identical protocolized regimens in immune suppression dosing and patient care, and were matched based on donor type and induction. Another limitation is the lack of association between age and infection in this cohort, due to the low overall rate of such events in our single center study. Extension of this study to a larger cohort size would allow us to achieve higher power to detect age-related differences in infection and death.

Future studies will involve larger cohorts of patients, analysis concomitantly with markers of T-cell immunity, and analysis of differential patterns of gene expression, including DNA methylation, to better understand the mechanisms of the observed differences in innate immune cell phenotype. Further subsetting of specific innate immune cell populations, such as the expression of activating versus inhibitory KIRs on NK cells, as well as evaluating innate immune cell function via in vitro stimulatory assays, will also help inform our knowledge of how the innate immune system changes with age and transplantation status. Similar analyses of innate immune cells from pretransplant samples will allow us to determine how posttransplantation immunosuppressive therapy differentially affects older and younger kidney transplant recipients.

By increasing our understanding of immune phenotypes in peripheral blood associated with clinical characteristics, such as patient age and donor type, as well as posttransplant events such as CMV viremia, we can begin to develop the ability to perform noninvasive immune monitoring of transplant recipients. This approach will lead to new strategies for risk stratification of older patients and individualization of immune suppression regimens to prevent infection and rejection after transplantation.

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